

Intracellular Signal Transduction and Amplification Mechanisms in the Regulation of Oocyte Maturation

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Abstract. Meiotic cell division can be induced by various extracellular signals in different organisms. The inducing signals interact with surface receptors. The signal is transduced across the oocyte plasma membrane, into the cytoplasm, where it is amplified by protein phosphorylation. Maturation-promoting factor appears in all meiotic and mitotic cells and is associated with protein phosphorylation. Cells use at least two systems for the activation of protein phosphorylation in response to extracellular stimuli: direct activation of a receptor-associated protein kinase and indirect activation via guanine nucleotide-binding proteins (G-proteins). G-proteins indirectly activate or inhibit protein kinases dependent on cAMP, Ca^{2+} , or diacylglycerol. Oocytes appear to use each of these signals and several different kinases to regulate meiotic cell division. The evidence for the involvement of each of these cellular signal transduction and amplification mechanisms in the regulation of meiotic cell division is discussed. Models are presented to account for possible interactions between various positive and negative modulators.

Introduction

Meiotic cell division is of fundamental biological importance. Meiosis is the process leading to the maturation of the egg and the generation of the haploid chromosome content necessary for the continuation of the species after fertilization. It can also be a useful model for processes involved in the regulation of mitotic cell division. Large populations of oocytes arrested at meiotic prophase can be obtained easily, are amenable to meta-

bolic labeling and biochemical analysis, and can be stimulated to undergo germinal vesicle breakdown (GVBD) synchronously without artificially synchronizing the population. Furthermore, meiotic cell cytoplasmic extracts cause breakdown of somatic cell nuclei. Thus, at least some of the intracellular regulatory processes leading to GVBD can also initiate mitotic cell divisions.

The reinitiation of meiotic maturation (removal of the prophase arrest) is normally triggered by an external effector stimulus. Often, but not necessarily, this stimulus is hormonal. The hormonal signals initiating GVBD in amphibian and starfish oocytes have been clearly identified as progesterone and 1-methyladenine (1-MA), respectively (for reviews see Kanatani, 1973; Masui and Clarke, 1979; Meijer and Guerrier, 1985). Amphibian oocytes can also be induced to undergo GVBD by exposure to insulin, which evidently acts by binding to an insulin-like growth factor receptor (IGFR) (Maller and Koonz, 1981). The hormones initiate GVBD by interacting with a component of the oocyte surface and are ineffective if introduced directly into the cytoplasm. Thus the hormonal signal must be transduced across the plasma membrane into the cytoplasm, where it elicits the biological response. In the cytoplasm, the signal can be positively or negatively modulated.

In other species, fertilization triggers GVBD, as well as egg activation and subsequent development. Sperm, of course, fertilize the egg by interacting with its surface. Thus it is reasonable to postulate that the transduction, relay, and amplification mechanisms involved in eliciting GVBD may be similar in oocytes induced by fertilization and hormonal stimulation. Furthermore, in the species in which the normal extracellular signal for GVBD is unknown, the oocyte may also use similar intracellular transduction and amplification mechanisms.

Invariably, there is a delay between the exposure of the oocyte to the stimulus for GVBD and its response. This delay may be several minutes or many hours long, depending on the species and the stimulus. A portion of this time period may be required for the generation of a sufficient number of hormone-receptor interactions (Bellé *et al.*, 1976; Masui and Clarke, 1979; Nemoto, 1982), but it is also followed by a period during which the hormone is no longer necessary, but prior to the onset of GVBD. In organisms such as *Urechis* and *Spisula*, in which fertilization provides the stimulus for GVBD, the time corresponding to the hormone-dependent period of starfish and frog oocytes is negligible since fertilization is a very rapid process. Yet there is a period of 3–4 min after fertilization during which the process of GVBD can be easily blocked, and this period is followed by another similar time period during which the process is essentially irreversible, but before GVBD (Tyler and Schultz, 1932; Allen, 1953; Carroll and Eckberg, 1986; Eckberg *et al.*, 1987). These delays suggest that time is required for the activation of intracellular signals and relays.

Recent research has begun to elucidate the mechanisms involved in transduction, relay, and amplification of signals in somatic cells. Most signals which elicit biological responses interact with receptors which are linked ultimately with molecules which regulate protein kinases or which possess protein kinase activity themselves. The former group includes protein serine/threonine kinases regulated by cyclic nucleotides, calmodulin (CaM), or diacylglycerol (DG) (see review by Edelman *et al.*, 1987). By contrast, members of the family of growth factor receptors have protein tyrosine kinase activity (see reviews by Hunter and Cooper, 1986; Hunter, 1987). The number of identified intracellular and membrane-bound protein kinases is increasing rapidly, and it has been proposed that they act as amplifiers and/or switches in many cellular responses (Hunter, 1987). Such coordinate and antagonistic action can provide a great deal of flexibility and sensitivity to the regulation of cellular responses. This article will discuss somatic cell transduction and amplification mechanisms and their relevance to mechanisms involved in eliciting GVBD. Specifically, it will discuss evidence for G-protein mediated signal transduction and the involvement of specific protein kinases in the initiation of oocyte maturation.

Maturation-promoting Factor and Protein Phosphorylation

Cytoplasmic events occurring during the delays result in the appearance of maturation-promoting factor (MPF), an operationally defined activity that is always found in oocytes prior to GVBD (see Masui and Clarke, 1979, for review). Its presence is defined by the ability of

the cytoplasm in which it is contained to cause recipient oocytes to mature rapidly and amplify the MPF (Masui and Markert, 1971). MPF is not tissue- or species-specific since an identical activity appears in all cells during the G₂ and M phases of the cell cycle (Kishimoto and Kanatani, 1976; Sunkara *et al.*, 1979; Kishimoto *et al.*, 1984; Dorée *et al.*, 1983; Miyake-Lye *et al.*, 1983; Gerhart *et al.*, 1984; Picard *et al.*, 1985b, 1987). Because this activity is present at mitosis, it is also called M-phase-promoting factor.

The widespread occurrence of MPF strongly indicates that it must play a fundamental role in the regulation of cell division. It may act generally to induce chromosome condensation and/or nuclear envelope breakdown (NEBD), since it can also cause NEBD, spindle formation, and chromosome condensation in somatic nuclei exposed to it *in situ* (Miyake-Lye *et al.*, 1983) and *in vitro* (Lohka and Maller, 1985). Although MPF can elicit these responses, it apparently does not do so directly because it can be separated from the factors directly responsible for these responses based on its failure to bind to nuclei *in vitro* (Newport and Spann, 1987).

MPF activity is invariably associated with protein phosphorylation. Microinjection of partially purified preparations of MPF causes an immediate increase in protein phosphorylation (Wu and Gerhart, 1980). Stage-specific protein phosphorylations occur in amphibian eggs and in cell-free extracts of amphibian eggs in response to MPF (Maller *et al.*, 1977; Karsenti *et al.*, 1987; Lohka *et al.*, 1987a).

MPF is stabilized by phosphatase inhibitors and labile in the presence of Ca²⁺ (Wu and Gerhart, 1980). Stabilization by phosphatase inhibitors indicates that the active form of MPF may be phosphorylated. The Ca²⁺ sensitivity could result from activation of a Ca²⁺-dependent protease or phosphatase. It could provide a mechanism for the disappearance of MPF after cell division.

Initial studies using gel filtration revealed an apparent molecular weight of roughly 100 kDa for MPF (Wu and Gerhart, 1980). Recently, it has been purified approximately 2000-fold from *Xenopus* eggs (Lohka *et al.*, 1987b). The fraction has protein kinase activity with broad substrate specificity and can cause nuclear breakdown and chromosome condensation *in vitro*. The fraction contains only two major polypeptides (45 kDa and 32 kDa) when assayed by SDS-PAGE. The 45 kDa polypeptide becomes phosphorylated in the presence of ATP. Whether the molecule with MPF activity that was partially purified by Wu and Gerhart is the same as that obtained by Lohka *et al.* has not been determined. It is possible that more than one macromolecule exists with MPF activity. Nevertheless, the close association of MPF activity with protein phosphorylation and its sensitivity to

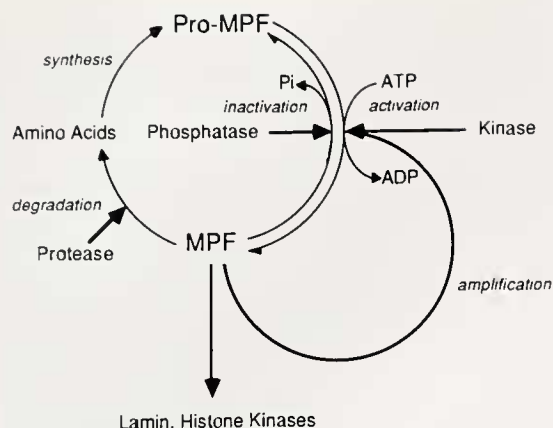


Figure 1. Scheme showing the proposed metabolism of MPF. MPF is synthesized as a precursor (Pro-MPF) which is activated by phosphorylation catalyzed by one or more protein kinases. It then amplifies its activity autocatalytically by autophosphorylation. It also activates other kinases (e.g., lamin and histone kinases) responsible for nuclear protein phosphorylation. It could be inactivated by dephosphorylation or by proteolytic degradation. Heavy lines terminating in arrows represent an activation process. Thin lines terminating in arrows represent chemical conversions. Italicized words indicate what is happening to MPF at each stage in the scheme.

phosphatases strongly indicates that MPF should be a protein kinase.

A scheme for the metabolism of MPF and its involvement in GVBD is shown in Figure 1. In this model, MPF is synthesized as an inactive precursor, Pro-MPF. It becomes active upon phosphorylation. MPF has kinase activity and amplifies its activity by autophosphorylation. It then activates other kinases which directly phosphorylate nuclear proteins, leading to nuclear envelope breakdown and chromosome condensation. MPF could be inactivated by dephosphorylation or by proteolytic degradation.

The mechanisms involved in the initial appearance of MPF remain to be explained. Clearly, though MPF can be amplified autocatalytically, its initial activity must be turned on or activated by some process derived from earlier events in the GVBD triggering process. Since MPF amplification is invariably associated with protein phosphorylation and MPF is stabilized by phosphatase inhibitors, its initial activation probably involves phosphorylation as well. It is not known which if any specific phosphorylation sites on the molecule are essential for its activation, but both protein tyrosine and protein serine/threonine kinases can lead to its activation. The protein tyrosine kinase activity of IGFR appears to be essential to its activity in inducing GVBD (Morgan *et al.*, 1986). The possible induction of GVBD by protein tyrosine phosphorylation is further supported by the observation that microinjected pp60^{v-src}, an oncogenic protein tyrosine kinase, can accelerate GVBD in *Xenopus* oocytes

(Spivak *et al.*, 1984). The other kinases that promote GVBD are protein serine/threonine kinases. A 42-kDa protein specifically phosphorylated at metaphase in *Xenopus* oocytes and oocyte extracts is phosphorylated on serine, threonine, and tyrosine residues (Lohka *et al.*, 1987a). This polypeptide has a molecular weight similar to that of a major phosphoprotein in highly purified MPF. The relationship between this molecule and MPF remains to be determined.

In principle, the disappearance of MPF activity could occur as the result of the action of either dephosphorylation or proteolysis. The stabilization of MPF by phosphatase inhibitors *in vitro* supports the idea that MPF is inactivated by dephosphorylation. However, the evidence suggests that dephosphorylation is not a general mechanism for the inactivation of starfish MPF *in vivo*. Co-microinjection of phosphatases into starfish oocytes does not block the amplification or activity of MPF (Meijer *et al.*, 1986). Furthermore, microinjection of the catalytic subunit of A-kinase, an antagonist of MPF production (see below), has no effect on the amplification or biological activity of MPF (Maller and Krebs, 1980). By contrast, cell cycle-specific proteolysis is known to occur (see below), and this activity may regulate MPF disappearance (Picard *et al.*, 1985b).

Protein phosphorylation "bursts" have been reported to precede GVBD in all species in which this phenomenon has been examined, including amphibians (Maller *et al.*, 1977), starfish (Guerrier *et al.*, 1977; Mazzei and Guerrier, 1982), molluscs (Eckberg *et al.*, 1987), echiuroids (Meijer *et al.*, 1982), and annelids (Peaucellier *et al.*, 1982, 1984). Some of the protein phosphorylation is undoubtedly due to the direct action of MPF, but other kinases are also implicated in specific protein phosphorylations. In some cases, these specific phosphorylations occur in response to MPF.

Ribosomal protein S6 is phosphorylated by two distinct kinases in *Xenopus* oocytes (Erikson and Maller, 1985, 1986; Erikson *et al.*, 1987) in response to hormonal stimulation or MPF microinjection (Hanocq-Quertier and Baltus, 1981; Nielsen *et al.*, 1982; Stith and Maller, 1984). There is no evidence that S6 phosphorylation has a causal relationship to GVBD. This ribosomal protein is phosphorylated in response to cell division and growth stimulating activities in a variety of cell types.

The phosphorylation of nuclear lamins and histones appears to be involved in GVBD and chromosome condensation. Lamin hyperphosphorylation occurs prior to NEBD (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985; Newport and Spann, 1987) and provides an essential condition for NEBD. The lamin kinase is distinct from MPF. There is a lag between MPF addition and lamin hyperphosphorylation (Miake-Lye and Kirschner, 1985), and MPF, unlike the lamin kinase, does not bind

to nuclei (Newport and Spann, 1987). Histone H1 phosphorylation also precedes GVBD (Hohmann *et al.*, 1976; Newport and Spann, 1987) and may provide an essential condition for chromosome condensation (Bradbury *et al.*, 1974; Inglis *et al.*, 1976).

G-proteins

Extracellular signals can regulate intracellular protein kinases indirectly by activating GTP-binding or G-proteins. A signal, the binding of a ligand to its cell surface receptor, causes the G-protein to exchange a bound GDP for GTP and become active by dissociation of the α from the β and γ subunits (Sternweis *et al.*, 1981). The activated α subunit may then regulate certain intracellular messenger systems which in turn regulate several specific cellular protein kinase activities indirectly. G-protein mediated cellular signal transduction has been recently reviewed (Stryer and Bourne, 1986). G-proteins are irreversibly activated by the binding of non-hydrolyzable analogs of GTP such as guanosine 5'-0-(3-thiotriphosphate) (GTP- γ S) and 5'-guanylyl imidodiphosphate (GppNHp) and are blocked from being activated by the binding of guanosine 5'-0-(2-thiodiphosphate) (GDP- β S). The substituted guanine nucleotides can act on any of the G-proteins. By contrast, cholera toxin (CTX) and pertussis toxin (PTX) antagonize specific G-proteins. Therefore, these compounds are also used to probe for G-protein function.

Mechanisms of G-protein regulation of cell function which may be relevant for GVBD are summarized in Figure 2. This scheme offers several potential levels of positive and negative regulation of GVBD. Adenylate cyclase is regulated by at least two separate G-proteins, G_s and G_i . The GTP-bound α subunit of G_s (45 or 52 kDa) stimulates adenylate cyclase, whereas the GTP-bound α subunit of G_i (41 kDa) inhibits the enzyme (Northrup *et al.*, 1983; Katada *et al.*, 1986). Hydrolysis of GTP to GDP returns the G-proteins to their inactive state. CTX ADP-ribosylates G_s , decreasing its GTPase activity and thus maintaining it in its active state (Cassel and Selinger, 1978). Similarly, PTX blocks agonist-mediated inhibition of adenylate cyclase by ADP-ribosylating the α subunit of G_i (Katada and Ui, 1982). By regulating the activity of adenylate cyclase, these G-proteins control the cellular cAMP content and thus regulate indirectly the activity of cAMP-dependent protein kinase (A-kinase).

Activation of another G-protein can activate a phosphatidylinositol-specific phospholipase C, resulting in the generation of inositol (poly)phosphates and diacylglycerol (Rodbell, 1980). The diacylglycerol activates a Ca^{2+} - and phospholipid-dependent protein kinase (C-kinase). The G-protein involved in this system could be

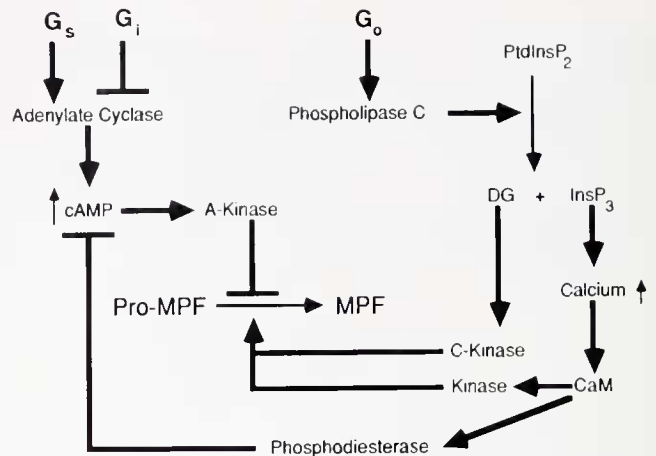


Figure 2. Possible regulatory mechanisms for G-proteins in GVBD. G_s and G_i regulate the content of cAMP, which regulates the activity of A-kinase, which antagonizes the conversion of Pro-MPF to MPF. G_o activates phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate (PtdInsP₂), producing diacylglycerol (DG) and inositol 1,4,5-trisphosphate (InsP₃). DG activates C-kinase which promotes the conversion of Pro-MPF to MPF. InsP₃ causes an increase in the cytoplasmic free calcium concentration due to release from intracellular stores. The elevated intracellular calcium activates calmodulin (CaM) which can activate a kinase which promotes the conversion of Pro-MPF to MPF, and/or it can activate phosphodiesterase which decreases the intracellular content of cAMP, thereby decreasing A-kinase activity. There is no direct evidence that MPF is a substrate for any specific kinase. However, it is not necessary for this model that the regulatory kinases act directly on Pro-MPF. There may be other intermediate substrates that act directly on Pro-MPF. In this and succeeding schemes, a heavy line ending in an arrow represents a stimulatory effect, a heavy line ending in a bar represents an inhibitory effect and a thin line ending in an arrow indicates a chemical conversion. Small vertical arrows adjacent to cAMP and Ca^{2+} indicate a cytoplasmic concentration increase.

G_o , the α subunit of which has an apparent molecular weight of 39 kDa. The distribution of G_o in mammalian brain parallels that of C-kinase (Worley *et al.*, 1986). Some of the inositol polyphosphates have Ca^{2+} -mobilizing activities, and the elevated Ca^{2+} concentration can activate other protein kinases, some of which are calmodulin (CaM)-dependent.

The evidence for the involvement of G-proteins in GVBD is strongest for frog and starfish oocytes. *Xenopus* oocytes have both the 45 and 52 kDa substrates of CTX and the 41 kDa substrate of PTX (Goodhardt *et al.*, 1984; Sadler *et al.*, 1984). In frog oocytes, CTX can block the induction of GVBD by progesterone (Godeau *et al.*, 1978). There is also some evidence that progesterone inhibits membrane-bound adenylate cyclase (Sadler and Maller, 1981) by blocking the action of a G-protein (Sadler and Maller, 1983). These data implicate the G_s in maintaining the oocyte in prophase meiotic arrest. They suggest that progesterone initiates GVBD by inactivating the G_s -protein, although the interpretation of some of

their data in this way requires novel mechanisms of G-protein action (Sadler and Maller, 1985). If this interpretation is correct, CTX should irreversibly activate oocyte adenylate cyclase *in vitro*. It might also be possible to introduce PTX to block agonist-mediated reduction in the cAMP content due to activity of the G_i protein. However, CTX does not antagonize the inhibition of *Xenopus* oocyte adenylate cyclase by progesterone *in vitro*, and PTX has little effect on GVBD (Goodhardt *et al.*, 1984; Sadler *et al.*, 1984). Thus the exact involvement of G-proteins in progesterone-induced *Xenopus* oocyte GVBD is unclear.

Other G-proteins can also mediate GVBD in amphibians. Microinjection of transforming *ras* proteins, oncogene products with the characteristics of unregulated G-proteins, can elicit GVBD (Birchmeier *et al.*, 1985; Deshpande and Kung, 1987; Lacal *et al.*, 1987), and microinjection of a monoclonal anti-*ras* can block both progesterone-induced (Sadler *et al.*, 1986) and insulin-induced GVBD (Deshpande and Kung, 1987). Some studies suggest that *ras* proteins may elicit GVBD by interacting with the adenylate cyclase system. For example, CTX partially blocks the action of microinjected *ras* (Birchmeier *et al.*, 1985). However, *ras* protein microinjection has no effect on the intracellular cAMP content (Birchmeier *et al.*, 1985), and other studies show that *ras* protein microinjection activates another G-protein-mediated signal system (Lacal *et al.*, 1987). Therefore, the best evidence indicates that *ras* proteins elicit GVBD by a mechanism that is independent of action on adenylate cyclase.

In starfish oocytes, microinjected GDP- β S inhibits hormone-induced GVBD (Shilling and Jaffe, 1987). Additionally, microinjected (Shilling and Jaffe, 1987) or externally applied (Eckberg, unpub.) PTX inhibits hormone-induced GVBD. PTX inhibition of GVBD could be mediated through action on either G_i or G_o , assuming that starfish G-proteins have responses to these toxins which are similar to those of mammalian cells.

There is also some evidence that G-proteins regulate GVBD in *Spisula* oocytes. Serotonin is a G-protein agonist in other systems (Litosch *et al.*, 1985), and serotonin can elicit GVBD in *Spisula* oocytes (Sato *et al.*, 1985). There is direct evidence for activation of a phosphatidylinositol-specific phospholipase C after fertilization (Eckberg and Szuts, 1987; Bloom *et al.*, submitted). However, preliminary experiments with PTX were inconclusive. Externally applied PTX had no effect on *Spisula* oocytes, but we could not demonstrate that the antagonist entered the oocytes (Eckberg, unpub.).

In *Chaetopterus*, the evidence for G-protein signaling in the initiation of GVBD is more indirect. Microinjection studies have not been performed on this organism, but fluoride, a G-protein agonist, can elicit GVBD at mi-

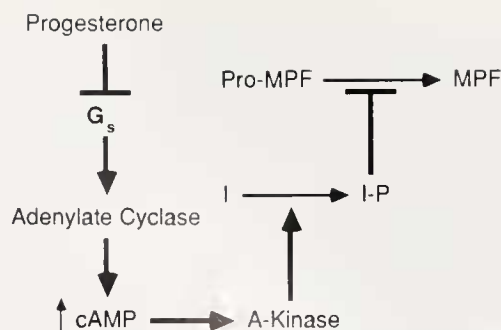


Figure 3. Possible scheme for induction of GVBD by progesterone in amphibian oocytes. Progesterone inactivates G_s which is coupled through adenylate cyclase and cAMP to A-kinase. A-kinase regulates GVBD by phosphorylating another molecule which inhibits the conversion of Pro-MPF to MPF. Thus the inhibitory action of A-kinase on the conversion of Pro-MPF to MPF and GVBD is blocked. Symbols are as in the legend to Figure 2.

cromolar concentrations (Eckberg, unpub.). While this evidence is weak by itself, there is evidence that products of G-protein-mediated signaling systems regulate GVBD in this species (see below).

Cyclic AMP

Cyclic AMP has been implicated in the maintenance of oocytes in meiotic arrest (reviewed by Maller and Krebs, 1980; Masui, 1985; Sadler and Maller, 1985; Maller, 1988). Amphibian oocytes show a temporary decrease in intracellular cAMP upon hormonal stimulation (Maller and Krebs, 1977; Speaker and Butcher, 1977; Sadler and Maller, 1985; Cicirelli and Smith, 1985; Maller, 1987). Furthermore, microinjection of the regulatory subunit of A-kinase promotes maturation, whereas microinjection of the catalytic subunit of the enzyme inhibits it (Maller and Krebs, 1977). Therefore, it is reasonable to suggest that progesterone elicits GVBD by inhibiting adenylate cyclase (Fig. 3). Recently, the evidence for such a model in *Xenopus* has been reviewed (Sadler and Maller, 1985; Maller, 1988). Nevertheless, it is not clear that the decrease in cAMP is sufficient to elicit GVBD in frog oocytes. Early reports indicated a large decrease in intracellular cAMP after progesterone addition (Maller and Krebs, 1977; Speaker and Butcher, 1977), but it is not clear how inhibition of adenylate cyclase can cause a large rapid decrease in the cellular cAMP content. Recently, the magnitude of the decrease has been disputed (Cicirelli and Smith, 1985). These last data indicate that a significant decrease in oocyte cAMP content may not be an obligatory step in the induction of GVBD by progesterone. Furthermore, although no data contradict such an interpretation, it has not been demonstrated definitively that a change in the cAMP concentra-

tion of the magnitude observed after progesterone treatment is sufficient to elicit GVBD.

In starfish, cAMP apparently *can* regulate GVBD, but it is unclear whether it does so *in vivo*. Microinjection of the catalytic subunit of adenylate cyclase may (Dorée *et al.*, 1981) or may not (Mazzei *et al.*, 1981) block 1-MA-induced GVBD, but microinjection of the regulatory subunit has no effect (Mazzei *et al.*, 1981; Dorée *et al.*, 1981). Initial investigations indicated that the oocyte cAMP content does not change in response to 1-MA treatment (Mazzei *et al.*, 1981; Dorée *et al.*, 1981), although recent studies indicate a gradual 10–30% decrease in the cellular cAMP content (Meijer and Zarutskie, 1987). However, it is unclear whether a decrease of this magnitude is a sufficient signal for GVBD, because treatment of the oocytes with forskolin, resulting in a 35-fold increase in the cellular cAMP content, does not block GVBD (Meijer and Zarutskie, 1987). In some other species, including *Spisula* and *Chaetopterus*, chemicals that elevate the cytoplasmic concentration of cAMP can inhibit GVBD (Sato *et al.*, 1985; Eckberg and Carroll, 1987), but there is no evidence that cAMP regulates GVBD *in vivo*. Similarly, CTX can block the induction of GVBD in *Xenopus* oocytes by insulin and *ras* proteins, agents which probably do not elicit GVBD by affecting intracellular cAMP levels.

To complicate this picture further, recent evidence indicates that an increase in the cellular cAMP content *causes* GVBD in the brittle star, *Amphipholis kochii* (Yamashita, 1988). This conclusion is based on the observations that chemicals that increase the cellular content of cAMP elicit GVBD and that the cellular cAMP content correlates positively with GVBD. Together these data demonstrate that intracellular levels of cAMP can regulate GVBD, but their involvement in the natural induction of GVBD varies between species.

Inositol Lipid Hydrolysis

Certain G-proteins transduce extracellular signals to the cytoplasm by activating a phosphatidylinositol-specific phospholipase C (Rodbell, 1980). This enzyme appears to exist in a membrane-bound (Cockroft *et al.*, 1984) and a soluble (Wilson *et al.*, 1984) form. The membrane-bound form is apparently regulated by a G-protein (Cockroft and Gomperts, 1985), whereas the soluble form is not. Both are Ca^{2+} -dependent and both hydrolyze phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) preferentially over phosphatidylinositol (PtdIns) at low free Ca^{2+} concentrations (Cockroft *et al.*, 1984; Wilson *et al.*, 1984). The membrane-bound form is apparently incapable of hydrolyzing PtdIns at physiological Ca^{2+} concentrations (Cockroft *et al.*, 1984).

Hydrolysis of all inositol lipids by phospholipase C re-

sults in the production of a membrane-bound second messenger, diacylglycerol, while the hydrolysis of each different inositol lipid also results in the production of a different water-soluble product, some of which have second messenger activity (reviewed by Nishizuka, 1984; Berridge and Irvine, 1984). Hydrolysis of PtdInsP_2 results in the production of inositol 1,4,5-trisphosphate (InsP_3), hydrolysis of phosphatidylinositol 4-phosphate (PtdInsP) results in production of inositol 1,4-bisphosphate (InsP_2), and hydrolysis of PtdIns results in production of inositol 1-phosphate (InsP). Of these, only InsP_3 has second messenger activity.

The most direct evidence for the involvement of inositol lipid hydrolysis in GVBD has been obtained in *Spisula* (Eckberg and Szuts, 1987; Bloom *et al.*, submitted) and *Xenopus* (Lacal *et al.*, 1987) oocytes. In *Spisula*, fertilization results in a loss of 25–35% of the radioactivity from both PtdInsP and PtdInsP_2 within 30 seconds after insemination. Thereafter, their specific radioactivities return to the preinsemination levels. These results clearly demonstrate that inositol phospholipid hydrolysis is a very early event after the fertilization of *Spisula* oocytes. Evidence that the products of this hydrolysis cause GVBD are presented below.

In *Xenopus* oocytes, inositol lipid hydrolysis follows the microinjection of transforming *ras* proteins (Lacal *et al.*, 1987). Microinjected *ras* proteins cause a significant disappearance of PtdIns and a significant production of InsP and diacylglycerol after a delay of a few minutes, with very little loss of PtdInsP and PtdInsP_2 or production of InsP_3 or InsP_2 (Lacal *et al.*, 1987). Since the earliest time point examined in this study was 2 min after *ras* protein microinjection, any rapid transient hydrolysis of PtdInsP_2 or PtdInsP comparable to that observed in *Spisula* would have been missed. Nevertheless, no diacylglycerol accumulated during the first two minutes, suggesting that there had been no hydrolysis of any inositol phospholipid during this time.

By contrast, PtdInsP and/or PtdInsP_2 hydrolysis appears to be necessary for insulin-induced GVBD because neomycin, a substrate-directed antagonist of phospholipase C, inhibits the response (Stiith and Maller, 1987). Neomycin binds preferentially to PtdInsP_2 and to PtdInsP , but has little binding capacity for PtdIns (Schacht, 1978; Whitaker and Aitchison, 1985). Neomycin inhibition of insulin-induced GVBD would thus implicate PtdInsP and PtdInsP_2 hydrolysis. Together these results implicate inositol lipid hydrolysis in *Xenopus* GVBD, whether induced by insulin or by *ras* proteins. The apparent involvement of polyphosphatidylinositol hydrolysis in insulin-induced GVBD is somewhat surprising, because it suggests that the IGFR may be linked to a G_o -like protein. If this interpretation is correct, it should be confirmed by direct measurement of inositol lipid pools

after exposure of the oocytes to insulin. Although fertilized *Spisula* oocytes and insulin-treated and *ras*-microinjected *Xenopus* oocytes all apparently use inositol lipid hydrolysis to elicit GVBD, the specific substrates preferred by phospholipase C in this response differ among them.

In *Chaetopterus*, there is only indirect evidence for this signaling mechanism in GVBD. Neomycin can block GVBD (Eckberg and Carroll, 1986). In other organisms, this pathway evidently has not been studied.

Inositol Trisphosphate and Calcium Release

Inositol 1,4,5-trisphosphate causes a direct release of Ca^{2+} from intracellular stores (Berridge and Irvine, 1984; Busa *et al.*, 1985; Clapper and Lee, 1985). InsP_3 can elicit responses from eggs. Sea urchin (Whitaker and Irvine, 1984; Turner *et al.*, 1986), frog (Busa *et al.*, 1985; Picard *et al.*, 1985a), and starfish (Picard *et al.*, 1985a) eggs all undergo a cortical reaction in response to InsP_3 microinjection.

In the starfish InsP_3 may not be involved in GVBD, because microinjected InsP_3 does not cause GVBD or prevent the oocyte from responding to 1-MA (Picard *et al.*, 1985a). Furthermore, despite many early observations which indicated involvement of Ca^{2+} release in the regulation of GVBD (see Meijer and Guerrier, 1985; Moreau *et al.*, 1985, for review), recent evidence indicates that Ca^{2+} transients are not required for 1-MA induced GVBD (Picard and Dorée, 1983; Eisen and Reynolds, 1984).

Similarly, microinjected InsP_3 does not elicit GVBD in *Xenopus* oocytes (Busa *et al.*, 1985; Picard *et al.*, 1985a). Nevertheless, microinjected InsP_3 can accelerate progesterone- or insulin-induced GVBD in *Xenopus* (Stith and Maller, 1987). If the effects of InsP_3 in this system result from Ca^{2+} release as expected, these observations indicate that elevated extracellular Ca^{2+} can facilitate GVBD, but is not a primary signal for it. Similarly, contrary to early reports (Moreau *et al.*, 1980; Wasserman *et al.*, 1980), a Ca^{2+} transient does not appear to be obligatory in the initiation of GVBD by progesterone (Robinson, 1985; Cork *et al.*, 1987). The cortical endoplasmic reticulum believed to be the source of the Ca^{2+} released at fertilization (Busa *et al.*, 1985) does not appear in oocytes until several hours after progesterone treatment (Charboneau and Grey, 1984). Thus, it is not surprising that InsP_3 does not elicit GVBD. The facilitating effect reported by Stith and Maller (1987) may be the result of a limited Ca^{2+} release from a rudimentary form of the cortical ER.

Spisula oocytes, in contrast to frog and starfish oocytes, undergo GVBD rapidly in response to microinjected InsP_3 (Bloom *et al.*, submitted). The effective con-

centrations (100% GVBD at a final intracellular InsP_3 of 60–80 nM) are very low and almost certainly must be exceeded as a result of the PtdInsP_2 hydrolysis that follows fertilization (Eckberg and Szuts, 1987; Bloom *et al.*, submitted). The results obtained clearly indicate that InsP_3 can elicit GVBD directly in this species. The discrepancy between these results and those obtained using frog and starfish oocytes are easily explained if one realizes that GVBD is triggered by fertilization in *Spisula*, and thus is a part of the egg activation process—a process that requires a Ca^{2+} flux in this and possibly all other species. Additionally, it must be remembered that *Spisula* oocytes undergo GVBD very quickly after fertilization (100% GVBD in >10 min). This rapid response may indicate that *Spisula* oocytes use signals not necessary in these other organisms.

Calmodulin

Calmodulin (CaM) is a small, highly conserved acidic protein that is involved in mediating the effects of Ca^{2+} on a number of enzymes (reviewed by Cheung, 1980). It is believed that CaM is present in excess in all cells and that it modulates cellular behavior by binding Ca^{2+} and then activating enzymes as a Ca^{2+} -CaM complex. Possible CaM involvement in regulating GVBD has been reviewed in more detail elsewhere (Meijer and Wallace, 1985).

CaM is present in oocytes, representing 0.2–0.5% of the cellular protein. It has been purified from frog (Cartaud *et al.*, 1980; Wasserman and Smith, 1981), starfish (Meijer and Guerrier, 1981), annelid (Carroll and Eckberg, 1983), and mammalian (Bornslaeger *et al.*, 1984) oocytes. CaM might promote GVBD by activating cyclic nucleotide phosphodiesterase. A CaM-dependent cyclic nucleotide phosphodiesterase has been reported in frog (Miot and Erneux, 1982) and mouse (Bornslaeger *et al.*, 1984) oocytes. Alternatively, CaM could elicit GVBD by activating a multifunctional CaM-dependent protein kinase (Stull *et al.*, 1986) (Fig. 2). Such enzymes have been demonstrated in frog (Wasserman and Smith, 1981) and starfish (Meijer and Wallace, 1985) oocytes. The existence of two reasonable mechanisms whereby CaM could regulate GVBD has made this protein an attractive system for study.

Several laboratories have studied possible CaM involvement in GVBD. These studies have followed two courses—microinjection of Ca^{2+} -CaM and microinjection or external application of CaM antagonists. Studies of the effects of microinjected Ca^{2+} -CaM on starfish oocytes have provided negative results (Dorée *et al.*, 1981; Meijer and Guerrier, 1981). In these studies, CaM neither inhibited nor facilitated GVBD when induced by 1-MA. By contrast, in frog oocytes microinjected Ca^{2+} -

CaM was reported to induce GVBD (Maller and Krebs, 1980; Wasserman and Smith, 1981). However, more recent studies were unable to confirm this finding (Cicirelli and Smith, 1987). The ineffectiveness of microinjected Ca^{2+} -CaM at inducing GVBD strongly suggests that CaM activation is not sufficient to elicit GVBD.

Antagonist studies, on the other hand, have provided evidence that CaM is necessary for GVBD in starfish (Meijer and Guerrier, 1981; Dorée *et al.*, 1982), *Chaetopterus* (Carroll and Eckberg, 1983), mouse (Bornslaeger *et al.*, 1984), and *Spisula* oocytes (Carroll and Eckberg, 1986). In frog oocytes, CaM antagonists evidently have different effects when they are applied externally than when they are microinjected (Cartaud *et al.*, 1980; Hollinger and Alvarez, 1982; 1984). Analogous results in starfish (Dorée *et al.*, 1981, 1982; Meijer *et al.*, 1981) oocytes led to the hypothesis that CaM is involved in the transduction of the stimulus for GVBD (Dorée *et al.*, 1982) or in some other early membrane-associated event. Such an hypothesis is also consistent with the observation that CaM antagonists only inhibit *Spisula* GVBD when applied within the first minute after fertilization, although Ca^{2+} is required for a longer time period (Carroll and Eckberg, 1986).

Several problems arise in trying to demonstrate a role for CaM in GVBD conclusively. First, CaM is activated by binding Ca^{2+} , and the evidence for a Ca^{2+} increase after induction of GVBD is controversial, as described above. Second, CaM is generally considered to be a soluble protein and has not yet been directly linked to any membrane-bound signal transduction mechanisms, so it is unclear how CaM could be involved in such transduction, although it could be involved in cytoplasmic amplification of transduced signals. Third, CaM antagonists are all known to affect other processes not dependent on CaM (see articles in Hidaka and Hartshorne, 1985), although some of the antagonists are more selective than others (*e.g.*, Mazzei *et al.*, 1984). In fact, evidence has been presented that certain CaM antagonists can block GVBD by a mechanism which is probably unrelated to CaM inhibition (Carroll and Eckberg, 1983). Fourth, the lack of a method for specifically activating CaM (other than by eliciting a cytoplasmic Ca^{2+} increase) makes it impossible to test directly whether CaM activation can cause GVBD. Thus, CaM may be involved in regulating GVBD, but if it is, the mechanisms involved are unclear.

Diacylglycerol and C-kinase

Inositol lipid hydrolysis by phospholipase C produces 1 mole of *sn*-1,2-diacylglycerol (DG) per mole of phospholipid hydrolyzed. Thus DG is the most abundant product of inositol lipid hydrolysis. DG has been identified as the physiological activator of C-kinase (see Nishi-

zuka, 1984, for a review). Tumor promoting phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), have also been shown to produce their cellular responses by binding to C-kinase and acting as a metabolically stable DG analog (Parker *et al.*, 1984; Tanaka *et al.*, 1986). A Na^+/H^+ antiport carrier is one substrate of C-kinase (Berridge and Irvine, 1984), and many of the effects of TPA on eggs can be explained as the result of activation of this enzyme and consequent cytoplasmic alkalinization (Swann and Whitaker, 1985; Lau *et al.*, 1986).

There is direct evidence that C-kinase activation necessarily precedes and can be sufficient to cause GVBD in *Spisula* oocytes (Dubé *et al.*, 1987; Eckberg *et al.*, 1987). The evidence includes several findings. First, TPA can elicit GVBD directly. Second, TPA elicits a pattern of protein phosphorylation similar or identical to that induced by fertilization. Third, C-kinase antagonists block GVBD whether induced by fertilization or by phorbol esters. Fourth, C-kinase activity can be demonstrated in oocyte extracts. Additionally Ca^{2+} and C-kinase apparently act synergistically in eliciting GVBD (Dubé *et al.*, 1987; Eckberg *et al.*, 1987). This also follows from the absolute Ca^{2+} dependence of *Spisula* GVBD and the ability of InsP_3 to elicit GVBD as well. Together these results strongly indicate that both products of inositol lipid hydrolysis act synergistically to elicit GVBD in this species (Bloom *et al.*, submitted). In this species, there is indirect evidence that the effects of TPA involve cytoplasmic alkalinization (Dubé, 1988).

In *Chaetopterus*, C-kinase activation appears to be both necessary and sufficient for GVBD (Eckberg and Carroll, 1987). However, the evidence for involvement of any specific kinase is indirect, because protein phosphorylation prior to GVBD has not been investigated. Phosphorylation precedes GVBD in another annelid, *Sabellaria* (Peaucellier *et al.*, 1982, 1984). Furthermore, the effects of TPA in this species do not appear to involve Na^+/H^+ exchange, because the effect is not dependent on extracellular Na^+ (Eckberg, unpub.).

Involvement of this pathway in GVBD does not appear to be restricted to invertebrates. TPA can elicit GVBD directly in frog oocytes (Stith and Maller, 1987). As with *Chaetopterus*, the effects of TPA do not depend on extracellular Na^+ . Together with the lack of effect of InsP_3 , these results suggest that DG is probably more important in regulating GVBD in the frog than the water-soluble products of inositol lipid hydrolysis. Furthermore, microinjected DG-activated C-kinase accelerates insulin induced GVBD, but has no effect on progesterone induced GVBD (Stith and Maller, 1987). These last results indicate that the metabolic pathways activated by C-kinase are also maximally activated by progesterone treatment, but not by insulin. Whether progesterone in-

duced GVBD depends on the activation of C-kinase apparently has not been tested.

Whether other organisms use this pathway is uncertain. Phorbol esters can block spontaneous GVBD in mouse oocytes (Urner and Schorderet-Slatkine, 1984; Bornslaeger *et al.*, 1986), and they can elicit GVBD in follicle-enclosed rat oocytes (Aberdam and Dekel, 1985), but the physiological significance of these observations is unclear. The culture conditions required to prevent spontaneous GVBD in denuded mouse oocytes are probably unphysiological, and in follicle-enclosed rat oocytes the cellular site of action of C-kinase agonists is ambiguous.

The situation in starfish oocytes is similarly confusing. A G-protein apparently transduces the hormonal signal, but InsP_3 does not elicit GVBD, and the involvement of the oocyte cAMP content in GVBD is controversial (see above). Therefore, DG might be expected to cause GVBD by activating C-kinase. However, exogenously added C-kinase agonists do not elicit GVBD (Eckberg, unpub.). Moreover, phorbol esters can block 1-MA induced GVBD (Kishimoto *et al.*, 1985). The significance of this inhibition is not clear, however, because very high concentrations of phorbol esters were used. The effective doses for inhibition of 1-MA-induced GVBD by phorbol esters are about two orders of magnitude higher than either their dissociation constants for purified C-kinase or the doses affecting GVBD in other organisms. Since inositol lipid metabolism has not been studied in starfish oocytes, it is unknown whether inositol lipid hydrolysis follows hormone addition or if this process is necessary for GVBD. Thus, while the involvement of G-proteins in starfish GVBD is very probable, the exact nature of the involvement has not been sufficiently examined.

Phosphatases

Given the importance of protein phosphorylation in GVBD, phosphatase activity would be expected to modulate GVBD. Indeed, microinjection of specific phosphatases inhibits hormone induced GVBD in amphibian (Hermann *et al.*, 1984) and starfish (Meijer *et al.*, 1986) oocytes, and microinjection of phosphatase inhibitors can directly elicit GVBD in starfish oocytes (Pondaven and Meijer, 1986). Phosphatase inhibition of GVBD could be overcome either by microinjection of MPF or by increasing the extracellular hormone concentration. However, microinjection of phosphatase 1 min before GVBD, a time *after* MPF should have appeared in the cytoplasm, still blocks hormone-induced GVBD (Meijer *et al.*, 1986). This last observation is difficult to reconcile with the ineffectiveness of phosphatase at inhibiting GVBD when induced by microinjected MPF. Thus, these data do not allow us to specify the site in the se-

quence of events leading to GVBD at which phosphatases act. While these data do not conclusively demonstrate a mechanism for regulation of GVBD by phosphatases, they provide further support for the importance of protein phosphorylation in the regulation of GVBD.

Genes That Regulate Mitosis and Meiosis

Cyclins are proteins that are synthesized at an apparently constant rate during the cell cycle, but are abruptly destroyed during mitosis (Rosenthal *et al.*, 1980; 1983; Evans *et al.*, 1983). Cyclins also cycle during the meiotic divisions of *Spisula* (Swensen *et al.*, 1986) and starfish oocytes (Standart *et al.*, 1987). One of the *Spisula* cyclin genes (cyclin B) has a protein kinase consensus sequence (Westendorf and Ruderman, 1987), and cyclins A and B both have potential phosphate acceptor sites (Swensen *et al.*, 1986; Westendorf and Ruderman, 1987). Additionally, microinjected cloned mRNA for *Spisula* cyclin A can drive frog primary oocytes through GVBD to meiotic metaphase (Swensen *et al.*, 1986).

These data indicate that the synthesis of a *Spisula* cyclin can activate GVBD in frog oocytes. Whether cyclins have a role in GVBD in other organisms is unclear. Clam cyclin A does not appear at detectable levels until after GVBD (Swensen *et al.*, 1986), although cyclin B is detectable in the prophase-arrested oocyte (Westendorf and Ruderman, 1987). The possible involvement of starfish cyclin (only one such protein has been reported in these oocytes, and the mRNA for this protein is at least partially homologous with clam cyclin A mRNA) in GVBD is unclear, because it was not assayed until after GVBD. High levels of cyclin do not correlate directly with NEBD in these oocytes, however, because this protein accumulates in the oocyte after the formation of the second polar body, even though the oocytes remain arrested in interphase until fertilization (Standart *et al.*, 1987). Similarly, the degradation of cyclins does not drive the nuclei into an interphase configuration because cyclins are degraded at both meiotic divisions, but no nuclear reformation occurs between the meiotic divisions (Longo and Anderson, 1970; Longo *et al.*, 1982).

The observation that high levels of cyclin do not necessarily correlate with NEBD in starfish and *Spisula* oocytes, even though cyclin mRNA can drive frog oocytes into M-phase, can be explained if cyclin protein must be posttranslationally modified to an active state. Frog oocytes have the ability to modify proteins appropriately, whereas the other oocytes may not. Since the cyclins have potential phosphate acceptor sites, it is reasonable to suggest that their activity could be regulated by phosphorylation. This interpretation predicts that the phosphorylation state of "active" cyclin present during the interphase/M-phase transition would differ from that

of "inactive" cyclin present during interphase. It also predicts that frog oocytes must rapidly modify the newly synthesized cyclin. An alternative explanation, that cyclin synthesis *does* drive GVBD in frog oocytes, is consistent with the fact that frog oocytes must undergo protein synthesis prior to GVBD, whereas oocytes of other species need not (reviewed in Masui and Clarke, 1979; Masui, 1985). The synthesis and/or postranslational modification of a cyclin may thus drive GVBD. In these interpretations, cyclin metabolism might be a common factor in the regulation of mitotic and meiotic cell division.

If mechanisms regulating mitotic and meiotic cell division are similar, then studies of the factors that regulate mitotic division should also have relevance to the study of meiotic cell division. Recently, a series of yeast genes regulating the passage through G_2 in the cell cycle has been reported. These include *cdc2*, *wee1*, and *nim1*, each of which encodes a protein serine/threonine kinase. The *wee1*⁺ protein is an inhibitor that delays entry into M until the cell has reached a certain size (Nurse, 1975). This protein is a 112 kDa protein kinase (Russell and Nurse, 1987a) which apparently inhibits the *cdc2*⁺ protein, a 34 kDa protein kinase (Hindley and Phear, 1984; Russell and Nurse, 1986) by phosphorylating it (Simanis and Nurse, 1986). Similarly, the *wee1*⁺ protein is inhibited by *nim1*⁺, a 50 kDa protein kinase (Russell and Nurse, 1987b). Thus a protein phosphorylation cascade regulates mitotic cell division in which mitosis occurs because an inhibitor of cell division is inhibited.

The *cdc2*⁺ protein is an activator of M phase, analogous to MPF. It can also be positively modulated by the protein product of the *cdc25*⁺ gene. The action of this gene product is required in addition to that of *nim1*⁺ for the cell to reach M-phase. If the *cdc25*⁺ protein proves to be a kinase, then phosphorylation would both positively and negatively regulate mitosis.

Conclusions, Model, and Prospects

It is evident, then, that intracellular signaling mechanisms similar or analogous to those involved in the regulation of somatic cell division and behavior are involved in the initiation of GVBD. It is also clear that, although protein phosphorylation is necessary for GVBD and is involved in the production and action of MPF, one single model cannot yet account for all of the data. Instead, kinases appear to regulate GVBD differently in different species and under different conditions. Therefore, we propose a model in which several different kinases act to promote or inhibit MPF activity, similar to the yeast model presented above. In our model, MPF is the central feature (Fig. 4).

Negative regulation of MPF is provided by A-kinase

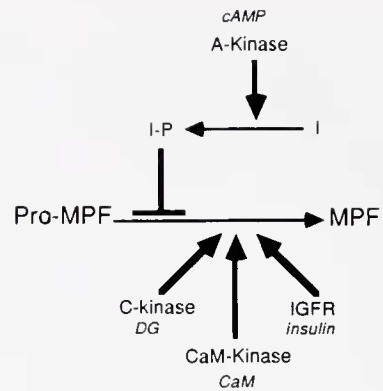


Figure 4. Proposed scheme for the interaction of kinases in the induction of GVBD. The cofactors for the active kinases are italicized. C-kinase, CaM-dependent kinase, and insulin-like growth factor (IGFR) each activates the conversion of Pro-MPF to MPF at a point downstream from the inhibitory effect of A-kinase. A scheme such as this allows the quantitative regulation of the conversion of Pro-MPF to MPF. Symbols are as in the legend to Figure 2.

in most species. In principle, A-kinase could inhibit MPF activity either directly, by phosphorylating a site on MPF which inhibits its activity, or indirectly, either by activating an inhibitor of MPF or by inhibiting an activator of MPF. The evidence indicates that this antagonism of MPF is indirect (see above and Fig. 3). The activity of A-kinase as an MPF inhibitor is regulated in turn by the cellular concentration of cAMP. Thus a decrease in cellular cAMP antagonizes the inhibition of MPF activity (*cf.* Fig. 3). An analogy in yeast mitosis is the inhibition of *wee1*⁺ protein by *nim1*⁺ protein.

By contrast, many other kinases promote the activation of MPF. These include C-kinase, CaM-dependent protein kinase, IGFR, and pp60^{v-src}. These could activate MPF directly, they could activate one or more other protein kinases that directly activate MPF, or they could inactivate an inhibitor of MPF, such as A-kinase or an inhibitory phosphorylated substrate of A-kinase. Inactivation of A-kinase would not seem to be a reasonable explanation for the induction of GVBD by C-kinase in *Chaetopterus*, because C-kinase activators elicit GVBD even in the presence of very high concentrations of cAMP (Eckberg and Carroll, 1987). Similarly, the catalytic subunit of A-kinase does not prevent the amplification of MPF (Maller and Krebs, 1980), indicating that A-kinase is not a direct inhibitor of MPF. Thus we propose that protein kinase activities that elicit GVBD do so by activating MPF, either directly or indirectly, downstream from the inhibitory action of the A-kinase substrate (Fig. 4). Thus the intracellular activators and inhibitors of meiotic cell division interact in a protein phosphorylation cascade that can regulate GVBD both positively and negatively. The existence of coordinate

and antagonistic protein kinase activities regulating GVBD would allow the oocyte maximum flexibility and sensitivity in responding to extracellular and intracellular signals regulating GVBD.

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